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PRINCIPAL INVESTIGATOR: Anthony A. Kossiakoff, Ph.D.
Charles V. Clevenger, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Chicago
Chicago, IL 69637

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Anthony Kossiakoff
Charles V. Clevenger

E-Mail: koss@bsd.uchicago.edu

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Chicago, IL 69637

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14. ABSTRACT

This proposal seeks to generate sABs (synthetic antigen binders) against the PRLr (prolactin receptor)signaling complex to systematically inhibit and modulate its important activities in breast cancer. This complex has clearly been demonstrated to play a significant role in development and spread of this disease, and yet the generation of pharmacologic agents that can specifically block their function has been slow. We hypothesize that sABs can be generated to the PRLr, CypA, and CypB proteins and delivered into the cell, where these reagents will block breast cancer growth, survival and spread. We have successfully generated sABs to CypA and B and the extracellular domain of PRLr and have begun their characterization. It is the intent of this proposal to validate the efficacy of these highly innovative reagents on a panel of breast cancer cells in vitro, with future translation into pre-clinical testing.

15. SUBJECT TERMS
Cyclophilin A, cyclophilin B, prolactin receptor, synthetic antibodies, phage display

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Introduction and Scope of Research: Our research objective is to develop a novel set of technologies that will target the Jak/Stat signaling cascade in breast cancer (1). These technol ogies, which involve a new class of affinity reagents and intramolecular delivery tools, have the capability of identifying the most important nodes in this signaling pathway and ultimately inhibiting or modifying them to influence effects on breast cancer cell proliferation and death. The interplay between the Jak and Stat com ponents in cytokine signaling has been an area of intense investigation. However, although many of the m olecular interactions that occur between the m and with other signaling partners have been broadly implicated in breast cancer, they are poorly characterized because of a lack of appropriate e xperimental tools. Consequently, a hos t of basic question s remain to be answered. Our goal is to develop an e xperimental framework to sort out the most important interactions in the pathway and establish whether there is a specific Achilles Heel that can be exploited to attack breast cancers in innovative ways. As a long-term goal, we will utilize this inform ation to develo p novel synthetic antibo dy reagents that can be delivered with precision and potency to breast cancer cells (2).

Research Accomplishments: Aim 1- Generating synthetic antigen binders (sABs) to components of the prolactin receptor signaling network. We picked three com ponents in th e prolactin signaling pathway to focus on using sAB technology to: 1) block binding of pr olactin (hPRL) to the extr acellular domain (ECD) of its cognate recep tor (hPRLr), 2) inhibiting CypA and 3) inhibiting CypB. CypA and C ypB are proline is omerase enzymes that play critical roles in signaling; in the case of CypA to s witch on the kinase activity of Jak2 and for CypB to assist in activation Stat5 in the nucleus (4, 5).

a) We have cloned, e xpressed and purified milligram quantities of CypA and C ypB. We will em ploy phage display mutagenesis to genera te sABs that inhibit the activity of these enzymes. W e have developed a sorting protocol that selects sABs th at block the ac tive sites of these enzymes using com petition with cyclosporinA (Figure 1). After round thre e, we picked 10 candidate sABs for each target (i.e. CypA and CypB) and evaluated their binding using surface plasm on resonance (SPR). sABs that bind to the e nzymes with Kds lower than 10 nM will be used in the biological assays described in Aim 2.

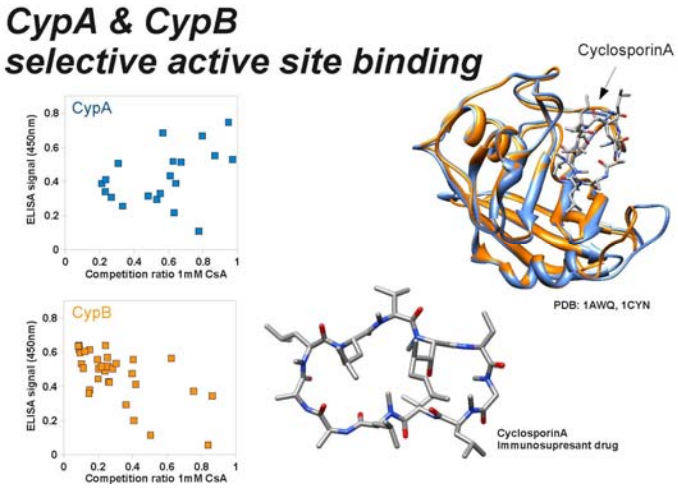

Figure 1- Phage ELISA data showing clones that interfere with cyclosporine binding indicating that they block the active site of the enzyme.

b) Using a novel phage display library (3), we have completed phage display selection for sABs that bind to the ECD of hPRLR at sites that inte rfere with hormone bindi ng. We theorized that this class of sABs will inhibit receptor signaling in cell-based assays by antagonizing hormone binding. Four candidate sABs that m et our criteria were evaluated for binding affinity using SPR. All the sABs had Kds less than 30 nM, suggesting that they had potential application as potent hPRLR antagonists. To understand the m echanism through which the sABs might block hormone-receptor bi nding, we chose one of the sABs to determine a high resolution X-ray crystal structure analysis of the hormone-receptor complex. The structure is shown below.

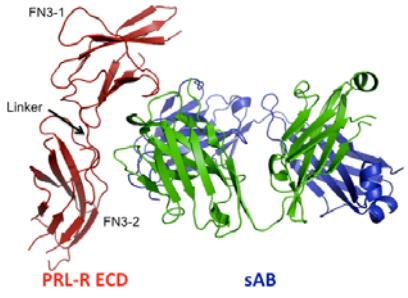

Figure 2- X-ray crystal structure of the hPRLR extracellular domain (ECD) bound to an inhibitory sAB. The receptor ECD has two fibronectin domains connected by a short linker. The sAB binds across the two domains. Interestingly, the known hormone bindi ng site is actually on opposite face of the ECD. Thus, antagonism is generated throug h indirect effects, not direct blocking of the h ormone binding site. The m echanism of horm one binding inhibition is based on the sAB altering the juxtaposition of the two fibron ectin domains in a way that changes the hormone binding site. We note, that ot her inhibitory sABs might work through other mechanisms, like directly blocking the hormone binding site.

Aim 2- We will use these inhibitory sABs in cell-based assays to evaluate their effectiveness as receptor antagonists. Our hypothesis is that sABs that block hormone binding will have an inhibitory effect on downstream signaling as measured by Stat activation. To assess this, we have tested four inhibitory sABs in cell based assays to measure inhibition of Stat5 activation (Figure 3).

Cells were incubated with increasing concentrations of each sAB, or a control sAB against bacterial maltose binding protein. The effect of the sABs on prolactin signaling was determined using a dual luciferase luminescence assay under the control of a phospho-Stat promoter (Figure 3A).

The results indicate that 3 sABs (A8, A9 and A10) significantly inhibit prolactin signaling in a concentration dependant manner.

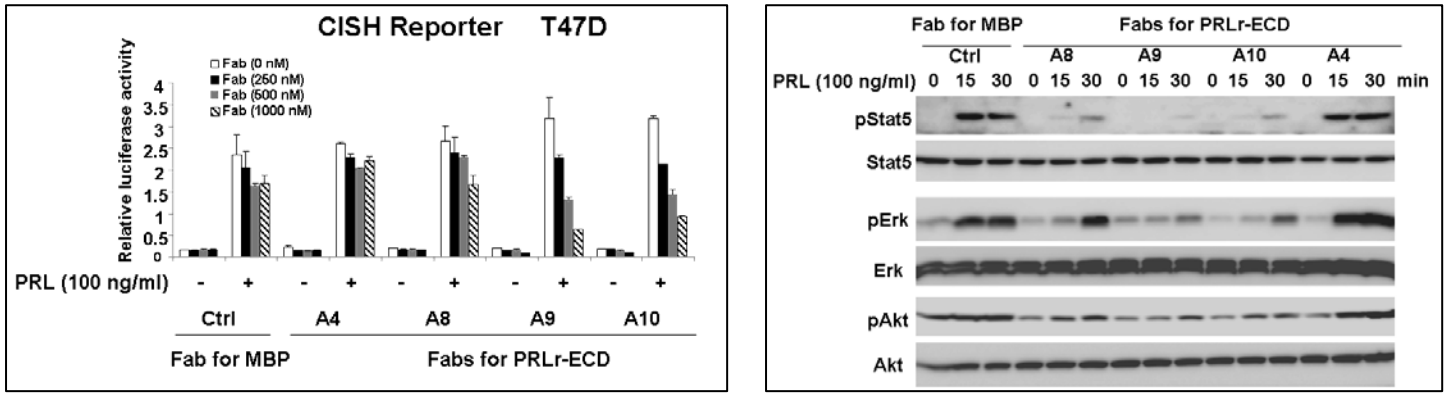

Figure 3- sAB inhibition of prolactin signaling. A) Effects on cell signaling based on luciferase expression. B) Measurement of decrease in phospho-Stat5 caused by the four individual inhibitory sABs.

To further examine the effect of the sABs on prolactin receptor signaling, we determined the change in the phosphorylation levels of various factors using western blot. While the control sAB (Fab for MBP) has no effect on prolactin signaling, sABs A8, A9 and A10 strongly inhibit phosphorylation of Stat5 and ErK (Figure 2B). Again, sAB A4 shows no effect on downstream signaling of prolactin as was observed in the luciferase assay.

sABs influence receptor internalization-

Many breast and ovarian tumors are characterized by the overexpression of the PRL-R. This triggers an overall enhancement of autocrine signaling through locally produced prolaction and down regulation of the signaling process by the internalization of the hormone-receptor complex. Thus, we examined the ability of the sABs to inhibit the internalization of prolaction and hGH through receptor endocytosis in T47D cells. This breast cancer cell line is known to express PRLR, which is rapidly internalized upon binding to hormone. T47D cells were incubated with 100 nM fluorescently-labeled prolactin or hGH in the presence or absence of 2 µM sAB A4, A8, A9 or A10 for 4 hrs. Fluorescence microscopy was used to investigate the effect of the sABs on the ability of the cells to uptake the hormones. The images indicate that sABs A8, A9 and A10 significantly decreased or completely abolished the internalization

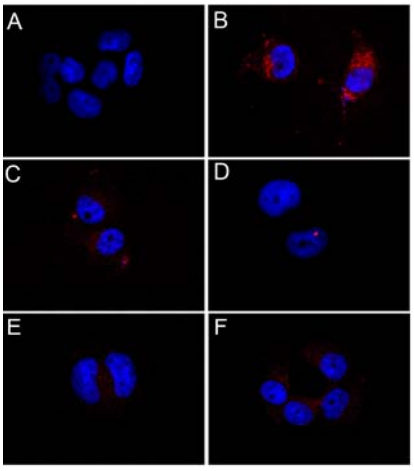

Fig. 4: Inhibition of internalization of prolactin by PRLR sABs. T47D cells were untreated (A) or treated with 100 nM prolaction-cy5 in the absence of sABs (B) or in the presence of 2 µM sAB A4 (C), sAB A8 (D), sAB A9 (E) or sAB A10. All panels represent two merged channels; blue: DAPI nuclear stain, red: cy5.

of prolactin, whereas sAB A4 had no effect (Fig. 4). sA B A4 also had no effect on the internalization of hGH (Fig. 5). In contrast with prolactin, hGH internalization was not completely inhibited by sABs A8, A9 and A10. This residual internalization of hGH is most likely carried out by the hGH receptor, which has been shown to be expressed in this cell line. Interestingly, sAB A9, which exhibits the highest affin ity for the receptor had the greatest inhibitory effect on the internalization of both hormones. sAB A4, does not bind to the receptor and had little to no effect on the internalization of either hormone

Key Research Accomplishments

- Used phage display to select sABs that act as inhibitors to CypA and CypB.
- Generated and characterized a sAB against the PRLr-ECD and showed it was a potent inhibitor against PRL binding to the ECD.
- Determined the X- ray crystal structure of the sAB- PRLr-ECD crystal structure at 2.8Å resolution identifying the mechanism of inhibition.
- Established that sAB binding interferes with PRL binding through an allosteric mechanism.
- Demonstrated that Receptor-mediated delivery can effectively deliver functional sABs into the cytoplasm.

Reportable Outcomes

Publications-

Rizk SS, Luchniak A, Brawley CM, Rock RS, Kossiakoff AA. (2009) "An engineered substance P variant for receptor-mediated delivery of synthetic antibodies into tumor cells." Proc Natl Acad Sci USA. 106:11011-5.

Duguid E, Zhang J, Rizk SS, Symborska A, Kouido J, Clevenger CV and Kossiakoff AA. " The structure and function of a synthetic antibody- complexed to the extracellular domain of the prolactin receptor: signaling inhibition through an allosteric mechanism" (in preparation).

Meetings where aspects of the work were presented through invited talks

Protein Society Meeting, San Diego, Ca.

ESF-EMBO Research Conference, Madrid, Spain.

Baxter Drug Discovery Symposium (keynote speaker) San Francisco, Ca.

University of Chicago Biosciences retreat Galena, Il.

Gordon Research Conference on Biomolecular Interactions and Methods, Galveston, TX.

9th Annual Antibody Therapeutics Conference, San Diego, CA.

Cold Springs Harbor Conference- Protein Structure based drug design, Suzhou, China.

CHI Symposium on Molecular Medicine, San Francisco, CA.

Seminar Presentations

Georgia Tech- Distinguished Lecture Series in Systems Biology

Oxford University

Johns Hopkins University Department of Biophysics

University of Maryland, Baltimore Departments of Biochemistry and Virology

University of Kansas, Department of Bioinformatics and Computational Biology

University of Texas Southwest Department of Biochemistry
Northwestern University, Department of Biochemistry, Molecular Biology and Cell Biology
Purdue University, Department of Biology
Genentech, Inc., Department of Protein Engineering
California Institute of Technology, Department of Chemistry
University of Wisconsin, Department of Biochemistry.

Patents

Patent Pending: "Receptor-mediated delivery of bioactive cargos" AA Kossiakoff inventor. (see appendix)

Conclusions

The reagents that are being generated are of exceptional importance to both basic and translational science in that they will provide a means of delivering inhibitory probes intracellularly into viable cells and modulate their activity.

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1 -Clevenger,C.V. Role of Stat family transcription factors in human breast cancer, Am J Pathol, *165:* 1449-1460, 2004.

2-Rizk SS, Luchniak A, Brawley CM, Rock RS, Kossiakoff AA. An engineered substance P variant for receptor-mediated delivery of synthetic antibodies into tumor cells, Proc Natl Acad Sci USA. 106:11011-5, 2009.

3- Fellouse,F.A., Esaki,K., Birtalan,S., Raptis,D., Cancasci,V.J., Koide,A., Jhurani,P., Vasser,M., Wiesmann,C., Kossiakoff,A.A., Koide,S. and Sidhu,S.S. High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries, J Mol Biol, *373:* 924-940, 2007.

4- Rycyzyn,M.A., Reilly,S.C., O'Malley,K. and Clevenger,C.V. Role of cyclophilin B in PRL signal transduction and nuclear retrotranslocation, Mol.Endocrinol., *14:* 1175-1186, 2000.

5- Rycyzyn,M.A. and Clevenger,C.V. The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer, Proc.Natl.Acad.Sci.USA, *99:* 6790-6795, 2002.

Appendix-

1) Patent description for receptor-mediated delivery technology

UChicagoTech

The University of Chicago
Office of Technology and Intellectual Property

Receptor-mediated delivery of large (up to 400 kDa) bioactive agents to live cells; can deliver antibodies, peptides, DNA, RNA and nanoparticles

A fundamental challenge in developing effective therapeutic agents for a wide variety of diseases is achieving efficient, specific delivery of the agents to the affected tissues. While significant advances have been made in the development of peptide, protein and DNA or RNA therapeutics, targeted delivery of these bioactive agents remains a major obstacle in drug development.

Technology Description
Dr. Anthony Kossiakoff and colleagues have developed a unique and highly effective technology for delivering bioactive agents to the cytoplasm of live cells without compromising the integrity of the cell membrane. This receptor-mediated delivery technology is based on an 11 amino acid variant of substance P (SPv), a neuropeptide that has nanomolar affinity for neurokinin receptor-1 (NK1R). Bioactive cargos can be conjugated to SPv and are efficiently internalized by NK1R expressing cells. The internalized cargos are able to escape from the endosome, but still retain their biological activity. Dr. Kossiakoff and colleagues have conjugated and successfully delivered a variety of different cargos to cells, including intact native and synthetic antibodies (sABs), other proteins and peptides, imaging agents, DNA, RNA (siRNA and shRNA), and nanoparticles (see Publication). Of particular note is that this technology can be used to deliver large protein payloads, up to 400 kDa, intact and functional to live cells.

Potential Benefit
NK1R is overexpressed in many types of tumors and primary cancers, including breast carcinomas, adenocarcinomas of the colon, astrocytomas, and glioblastomas. Consequently, this technology could have great therapeutic potential by allowing one to discriminate between cancer cells expressing the NK1R and normal cells. Further, the specific delivery of sABs directed toward cytoplasmic targets within tumor cells potentially changes the paradigm for antibody-based therapies. These therapies may no longer be limited to the current extracellular targets. Thus, using sABs designed to inhibit intra-cellular signaling nodes, the possibility certainly exists for focusing future antibody therapies toward a much richer set of cancer targets.

Product: Pharmaceutical (delivery platform)

Development Stage: Optimize Lead / *in vitro* Testing

Primary Inventor: Anthony Kossiakoff PhD, Ortho S.A. Sprague Professor and Chair, Biochemistry & Molecular Biophysics

Scientific Publication: Rizk *et al. PNAS.* 2009 Jul 7; 106(27): 11011-5

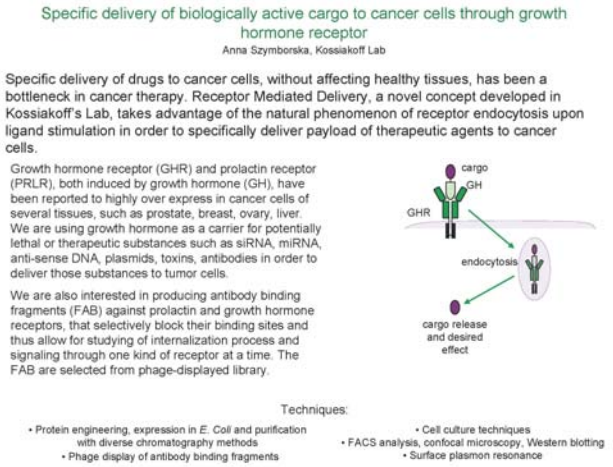
License Status: Available for licensing

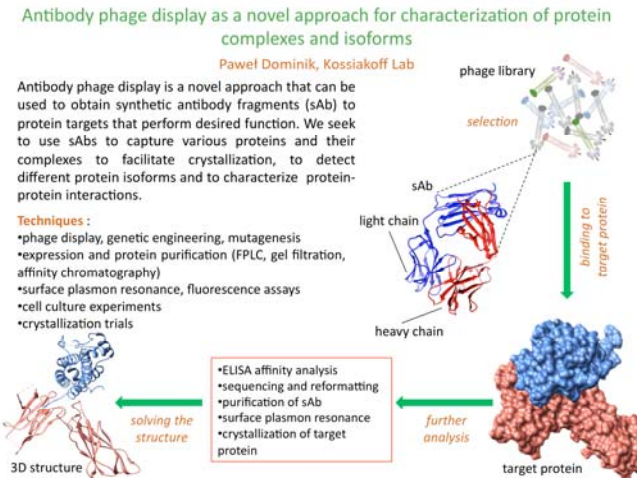
Patent Status: 12/375,179 pending (PCT WO 2009/076463 A1)

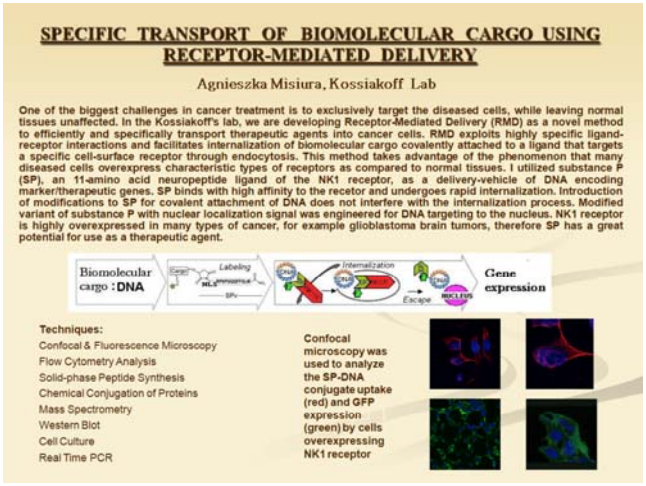
Reference: UCHI 1670

Contacts: Ben Dibling PhD, bdibling@tech.uchicago.edu, (773) 834-1270,
or Thelma Tennant PhD, ttennant@tech.uchicago.edu, (773) 834-4020

2) Student posters at Biosciences Retreat Galena, IL 11/10- 11/11.







Supporting Data- None